

Phylogenetic Study of Dioecious and Parthenogenetic Populations of *Canthocamptus staphylinus* (Crustacea, Copepoda, Harpacticoida)

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Abstract.—The phylogenetic relationships of four dioecious populations and one parthenogenetic population of the harpacticoid *Canthocamptus staphylinus* (Jurine, 1820) were studied. Analysis of the mtCOI gene revealed two main clades as a phylogenetic tree and a network of haplotypes: a clade with Fennoscandian populations in Lake Pääjärvi (Finland) and Lake Vänern (Sweden), and a second clade with populations in Lake Võrtsjärv (Estonia), Orlov Pond in Saint Petersburg (Russia), and the type locality of the species in Lake Geneva (Switzerland). The parthenogenetic population of *C. staphylinus* showed the smallest nucleotide and haplotype polymorphisms and could have evolved as a reaction to the changing environmental conditions following the Last Glacial Maximum, 20K YBP.

The harpacticoid copepod *Canthocamptus staphylinus* (Jurine, 1820) is one of the most widely distributed freshwater harpacticoid species in the Palearctic region (Lang 1948; Borutzky 1952; Fefilova 2015). Normally, it is a stenothermal and psychrophilic form and, according to different sources, inhabits waterbodies with temperatures ranging from 10° to 19°C (Sarvala 1979a). As part of the life cycle, adult *C. staphylinus* rests encysted in the bottom mud. Discovery of the species in different types of water bodies — from small spring pools to large lakes and rivers — indicates its high environmental plasticity. Individuals are both geographically and ecologically variable in several morphological characteristics, including the structure of the fifth pair of thoracal legs, numbers of spinules on the anal operculum, form of the spermatophore, and the development of the aesthetascs borne on the fourth segment of the antennule (Lang 1948; Borutzky 1952; Fefilova 2015). This variability suggests that the forms described as *C. staphylinus* in the literature may actually represent a group of closely related species.

Breeding of harpacticoid copepods is commonly dioecious. They possess clear sexual dimorphism — females are usually larger than males and have different morphologies of antennules and swimming legs (Borutzky 1952; Huys and Boxshall 1991; Suárez-Morales 2015). However, parthenogenetic reproduction has been verified in a few harpacticoid species. The first reports were for *Elaphoidella bidens* (Schmeil, 1894) and *Epactophanes richardi* Mrazek, 1893, both from the family Canthocamptidae. For *C. staphylinus* a parthenogenetic life cycle was proved to occur in the stock living in the oligotrophic Finnish Lake Pääjärvi. There are both field data (males comprised only 0.28% of adults) and laboratory observations (unmated females isolated as nauplii or copepodids and reared individually in the laboratory, produced viable offspring) (Sarvala 1979a). In

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Table 1. *Canthocamptus* spp. sampling sites.

No.	Country, area	Locality	Coordinates	Number of individuals	Abbreviations
1	Geneva, Switzerland	Lake Geneva	46°27'N, 06°31'E	8	Geneva
2	Hämeenlinna, Finland	Lake Pääjärvi	61°04'N, 25°08'E	11	Paajarvi
3	Estonia	Lake Võrtsjärv	57°40'N, 26°40'E	5	Vortsjarv
4	St. Petersburg, Russia	Orlov Pond	59°51'N, 30°02'E	10	SPB
5	Sweden	Lake Vänern	58°55'N, 13°30'E	9	Vanern
6	Syktyvkar, Komi Republic, Russia	A pond in the Botanical Garden	61°40'N, 50°49'E	1	<i>C. microstaphylinus</i>

addition, parthenogenesis has been suspected on the basis of the scarcity of males in some other *Elaphoidella* species: *E. leruthi* Chappuis, 1937 and *E. elaphoides* (Chappuis, 1924) in the northernmost part of their range (Chappuis 1955), and *E. grandidieri* (Guerne and Richard, 1893).

For different types of invertebrate animals, a parthenogenetic life style serves as an adaptation to unfavorable environmental conditions (Clark and Bowen 1976; Glesener and Tilman 1978; Grebelnyi 1996; Hebert and Finston 2001; Hebert et al. 2007; Weeks et al. 2008). Asexual or parthenogenetic organisms occur more often at higher latitudes and altitudes, on islands and in environments variously classified as marginal, stressful, transient or disturbed (Cuelar 1977; Glesner and Tilman 1978; Bell 1982; Lynch 1984; Suomalainen et al. 1987). Although clonal reproduction is expected to be reproductively advantageous (Maynard-Smith 1978; Bell 1982), obligately asexual organisms are short-lived on geologic time scales and have been regarded as evolutionary dead ends (Bell 1982; Lorenzo-Carballa et al. 2012.). However, in the short term, switching to an asexual mode can help organisms to exploit a narrow spectrum of environmental resources more efficiently than could genetically diverse sexual populations (the “demographic balance” hypothesis, Gets 2001; Haag and Ebert 2004). Molecular responses to environmental changes are modulated by phenotypic plasticity (physiological acclimatization) and genetic adaptation (genetic evolution through natural selection under new environmental conditions) and may play important roles in the persistence of the species (Smolina 2015). To reveal the phylogenetic relationships among parthenogenetic and dioecious populations of *C. staphylinus* we studied the genetic population structure of this species across a wide geographic area using mitochondrial DNA sequence data.

Material and Methods

Samples were collected from 2015 to 2016 from four dioecious populations of *C. staphylinus* (including the type locality of the species in Lake Geneva, Switzerland) and one parthenogenetic population in Lake Pääjärvi. In addition, a population of *Canthocamptus microstaphylinus* Wolf, 1905 was sampled in 2015 in Syktyvkar, Komi Republic, Russia and used as a group for comparison with closely related species. Samples from the Orlov pond and Lake Geneva were collected by Natalia M. Sukhikh in November 2010 (Table 1, Fig. 1). All samples were collected close to the shore with a hydrobiological



Fig. 1. Sample locations of specimens of *Canthocamptus* spp. examined in this study. Locality names are listed in Table 1.

100- μ m mesh hand net. Organisms in these samples were preserved in 96% alcohol solution. Morphological identification of species was performed using an ES Bimam R 13-1 Microscope (Russia), and Micromed MC-4-Zoom Led stereoscopic microscope (Russia) according to the descriptions by Lang (1948).

A 611 base pair (bp) fragment of the mitochondrial cytochrome oxidase I (mtCOI) gene was sequenced from 44 adult copepods. Genomic DNA was extracted from copepods preserved in 96% ethanol following protocols outlined in Walsh et al. (1991). Specifically, the bodies of each crustacean were added to 5% Chelex-100 (Sigma-Aldrich, St Louis, MO, USA) solution in bidistilled water. Samples were spun for 30 s at 12,000 rpm. The mixture was incubated at 90°C for 30 min, then spun for 30 s at 12,000 rpm. Samples were incubated at 90°C for 15 min and spun for 15 s at 12,000 rpm. Then samples were stored at -20°C and amplified through PCR. Universal primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) were used for amplification of COI by polymerase chain reaction (PCR). PCR started with DNA denaturing at 95°C (60 s), followed by 35 cycles of 30 s denaturing at 95°C, 30 s annealing at 50°C, and 50 s extension at 72°C, and then a final extension at 72°C for 7 min (Lee 1999). The product was purified with a Qiaquick PCR purification kit and sequenced in an ABI Prism 310 automated sequencer. To avoid mistakes in sequences we used bi-directional sequencing

Table 2. Percent genetic distances for the six populations of *Canthocamptus* spp. based on mtCOI sequences.

Population	Pääjärvi	Vänern	SPB	Vörtsjärv	Geneva
Pääjärvi					
Vänern	2.5				
SPB	22.5	22.1			
Vörtsjärv	23.5	23.7	3.1		
Geneva	23.4	23.1	2.1	1.2	
<i>C. microstaphylinus</i>	25.5	27.0	26.8	26.2	26.6

with above mentioned primers. In case of double peaks or ambiguous base calls each sequence was compared with obtained chromatograms, a signal that exceeded the other was taken into account. All processing was done at the Center for Collective Use “Molecular Biology” of the Institute of Biology, part of the Komi Scientific Center of the Ural Branch of the Russian Academy of Science.

Nucleotide sequences were aligned with the algorithm CrustalW and corrected manually (due to several shifts of nucleotide motifs after alignment) using the program package Geneious (version 7.0.6.) (Kearse 2012). Phylogenetic trees were also constructed in Geneious using a Maximum Likelihood method with a high level of the bootstrap coefficient (1000 replications). A median network of haplotypes was constructed with Network 4.6.1.3 (Bandelt 1999). Statistical analysis of the DNA polymorphism was performed in DNAsp 5.10 (Librado 2009). All sequences of *C. staphylinus* were registered in GenBank under the accession numbers KP974713–KP974719 and MG209708–MG209737. In addition, a sequence of *C. microstaphylinus* (accession number KP974734.1) sampled and sequenced according to methods described above was included in the phylogenetic analysis. For comparison, three further sequences obtained from GenBank were added: *C. staphylinus* from Northern Germany (MF077881.1), *Canthocamptus coreensis* Chang, 2002 (KT030277.1) and *Elaphoidella humphreysi* Karanovic, 2006 (JN039173).

Results

A total of 43 *C. staphylinus* females were sequenced for the mtCOI gene. Males were observed in all populations except the parthenogenetic one in Pääjärvi. Moreover, some of the females in Pääjärvi were bearing egg sacs but had no spermatophores attached to their bodies, suggesting that mating had not taken place for these individuals.

Phylogenetic analysis revealed two strongly separated clades with maximum pairwise divergences of 23.7% between geographically separate populations (Fig. 2, Table 2). All clades were strongly supported by bootstrap percentages and were divided mostly according to the populations’ geographical locations. The clade I was common for three populations of the species *C. staphylinus*: the type locality in Lake Geneva, Orlov Pond in Saint Petersburg and Lake Vörtsjärv. These three populations formed three main groups that roughly but not completely followed their geographical origin with genetic divergences between them varying from 1.2% to 3.1%. Two sequences of *C. staphylinus* from Orlov pond were placed in the group for Lake Geneva sequences, while two sequences of the species from Lake Geneva fell into groups for Lake Vörtsjärv and Orlov pond. The clade II included two subclades, consisting of the parthenogenetic population from Lake Pääjärvi and the dioecious population from Lake Vänern together with the sequence of

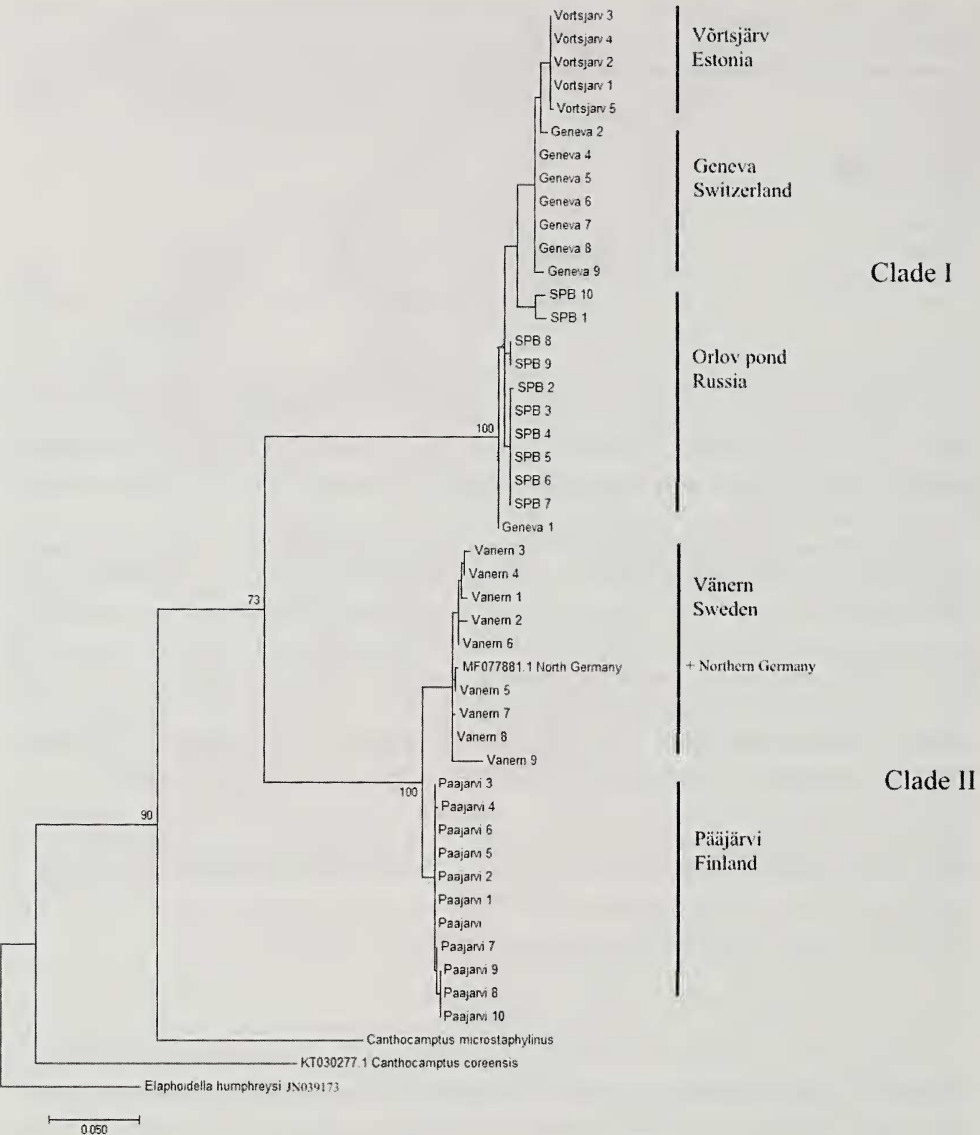


Fig. 2. Phylogenetic tree of the relationship between *Canthocamptus staphylinus* populations based on data from mitochondrial cytochrome oxidase subunit I (COI) region (611 bp) by the maximum likelihood method. Numbers beside nodes indicate bootstrap values. As an outgroup, a sequence of *Elaphoidella humphreysi* from NCBI was used (accession number JN039173). From GenBank, a sequence of *C. staphylinus* from northern Germany (accession number MF077881), a sequence of *Canthocamptus microstaphylinus* (accession number KP974734.1), and a sequence of *Canthocamptus corensis* (accession number KT030277.1) were also included.

C. staphylinus sampled in Northern Germany with genetic divergence of 2.5% between them.

The COI haplotype network formed two separate clades similar to those distinct in the phylogenetic tree (Fig. 3). The total number of haplotypes was 22. The *C. staphylinus* clade II consisted of the parthenogenetic population from Pääjärvi and

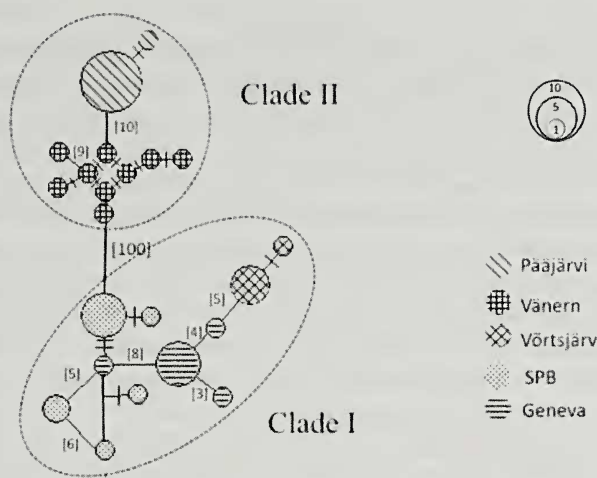


Fig. 3. Haplotype network based on statistical parsimony, representing genealogical relationships between mtCOI haplotypes in sexual and parthenogenetic populations of *Canthocamptus staphylinus*. Numbers in square brackets and hash marks represent mutations between haplotypes. The size of the circles correlates with haplotype frequency. The dashed circles in the network indicate the three main clades with a significant geographic association (see the main text).

the dioecious population from Vänern. The most frequent haplotype 1 in the haplotype network belonged to the parthenogenetic population. The clade I was formed of the *C. staphylinus* populations from Lake Geneva (the species type locality), Orlov Pond in Saint Petersburg and Lake Vörtsjärv. The latter clade held a central position in the haplotype network, with all other haplotypes extending away from it. Numbers of mutations were 100 between the first and the second clades.

Analyses of the sequence polymorphism of the populations allocated to *C. staphylinus* are summarized in Table 3. Among 43 obtained sequences, there were 22 haplotypes, and 135 polymorphic sites with 147 mutations. General haplotype and nucleotide diversities were 0.920 and 0.1067, respectively. The haplotype and nucleotide diversities observed in the parthenogenetic population were lower than in the dioecious populations. The smallest numbers of polymorphic sites and mutations were detected in populations from Lake Pääjärvi and Lake Vörtsjärv.

Table 3. Polymorphism of the COI gene from *Canthocamptus staphylinus* populations. n — number of sequences, N — Number of haplotypes, Hd — Haplotype diversity, L — length of sequences, Pi — Nucleotide diversity, S — Number of polymorphic (segregating) sites, Eta — number of mutations. The parthenogenetic population is indicated in bold.

Population	n	N	Hd	L	Pi	S	Eta
Pääjärvi	11	2	0.182	611	0.00033	1	1
Vänern	9	9	1.000	611	0.00884	18	18
SPB	10	5	0.756	611	0.01118	20	20
Vörtsjärv	5	2	0.400	611	0.00066	1	1
Geneva	8	4	0.643	611	0.00702	17	17

Discussion

The demographic advantage of obligate parthenogenesis for aquatic invertebrates is the possibility of accelerating the life cycle during a short period of favorable conditions (Lynch and Gabriel 1983; Hebert and Hann 1986). In copepods, parthenogenetic reproduction has remained poorly known, reflecting that it is a rarity in nature and uncommon for this taxon. As was mentioned above, in a few species of harpacticoids of the family Canthocamptidae parthenogenesis has been suspected because males are rarely found, and in some of those species laboratory experiments show that females can reproduce by themselves without males (Sarvala 1979a; Dole-Oliver et al. 2000; Gutierrez-Aguirre et al. 2011). Across the distributional range of *C. staphylinus*, only one parthenogenetic population is known, that from Lake Pääjärvi (and possibly another population at 80 km distance from it). All other populations studied, including those of the present work, were dioecious with males present in every population (Smyly 1957; Sarvala 1979a; Dole-Oliver et al. 2000; Fefilova 2015). The parthenogenetic population of *E. richardi* was also described from the Scandinavian region. Moreover, *E. richardi* var. *angulatus* Kulhavi, 1957 hatched out from parthenogenetic eggs, was able to develop and reproduce sexually with sperm transfer from males to females (Lang 1935; Borutzky 1952).

Many species of copepods have female-biased adult sex ratios (Kjørboe 2006). A variety of factors have been suggested to influence the sex determination in this taxon: temperature (Katona 1970); food (Irigoin et al. 2000); population density (Heinle 1969) and pressure (Vacquier and Belser 1965). For example, in *Calanus* spp. food quantity and quality have a significant effect on the seasonally changing population sex ratio, supporting the existence of a strong environmental influence on sex determination (Svensen and Tande 1999; Irigoin et al. 2000; Miller et al. 2005; Gusmão 2009).

Our analysis of genetic population structure reveals a broad range of genetic divergence (1.2–23.7%) among populations of *C. staphylinus* (Fig. 2, Table 2). The genetic divergence between clades of haplotypes also was large and generated a haplotype network with its branches containing many mutations between populations (Fig. 3). Interestingly, the genetic divergence between the two clades of *C. staphylinus* was similar to that between *C. staphylinus* and closely related but separate species of *C. microstaphylinus* (more than 20%). This suggests the existence of a complex of at least two cryptic species among the *C. staphylinus* populations. This complex would include a “northern” species with populations from Finland, Sweden and Northern Germany, and a “continental” species with populations from Russia, Estonia and Switzerland (Fig. 2).

Similar unexpectedly wide genetic variability revealing the existence of several cryptic forms has been found in other studies of molecular-genetic population structure of harpacticoids. For example, in a study of *Nannopus palustris* Brady, 1880 genetic distances of mtDNA reached up to 78% between populations and allowed to separate several cryptic forms (Garlitzka et al. 2012). According to studies on phylogenetic and phylogeographic structure among populations of *Tigriopus californicus* (Baker, 1912) divergences of mtDNA often exceeded 20% (Burton et al. 2007; Willet and Ladner 2009). High levels of intra- and interspecific divergence seem to be widespread phenomena among members of the order Harpacticoida (Schizas et al. 1999; Easton et al. 2010). In freshwater copepod species, the observed molecular genetic divergence can be the result of founder effects accompanied by limited gene flow between populations, even those in adjacent habitats (Bucklin 1998).

The pattern of population structure which is observed in *C. staphylinus* is defined by the present-day gene flow as well as the historical processes. The clade which consists of the

Pääjärvi and Vänern populations (Fig. 2) suggests that they have a common history. Indeed, during the development of the Baltic Sea after the last Ice Age, the basins of Pääjärvi and Vänern were both parts of the Yoldia Sea stage (10300–9500 years BP; Björck 1995), the water of which was fresh for most of the time. While Lake Pääjärvi was already isolated from the Yoldia Sea (Okko 1969), Lake Vänern became separated slightly later, at about 9500 years BP, just at the transition to the following Baltic freshwater stage, the Ancylus Lake. Hence, the animal populations of these lakes would have close relationships.

The unexpected clear distinction between the Swedish and Finnish populations on one hand, and the geographically rather close Vörtsjärv and Saint Petersburg populations on the other hand, indicating long isolation of these populations, also becomes understandable on the basis of existing data on the geological history of the area. The continental ice started to retreat from Estonia around 13000 years BP and had disappeared by 11000 years BP (Moora et al. 2002). There was an Ice Vörtsjärv up to 12200 years BP, partly dammed by ice, followed by Big or Ancient Vörtsjärv during 12200–7500 or 7000 years BP, after which there has been the contemporary lake. Vörtsjärv was thus from the beginning separated from the successive stages of the Baltic basin. The same applies to most of the Saint Petersburg region. The distance between Vörtsjärv and Saint Petersburg is not so great, around 700 km, and resting stages in the form of cysts might allow more efficient dispersal than is the rule in harpacticoids.

The similarity of the populations from Vörtsjärv and Saint Petersburg with that from Lake Geneva (Figs. 2 and 3) is more difficult to explain on the basis of existing geological information. The overlapping structure of the distribution of sequences in the phylogenetic tree and the haplotype network together with relatively small genetic differences in spite of considerable geographical distances suggests that these populations might have an ancient history of dispersal long before than the last Ice Age. The relationships between these harpacticoid populations represent a similar problem as the present-day distributions of the so-called “glacial relicts” in and around the Baltic Sea, which have been much discussed relative to the improving knowledge of the geological history (Segerstråle 1982). Even in case of the “glacial relicts” the explanations have remained speculative.

A detailed analysis of the distribution patterns of dioecious and parthenogenetic forms of insects was carried out by Suomalainen and collaborators (Suomalainen and Saura 1973; Suomalainen et al. 1976, 1987). It proved that the invasion of polyploid populations (which are common for asexual organisms like hermaphrodites and parthenogens) occurred as a result of the Quaternary Glaciations. These polyploid insect populations are supposed to have originated from diploid populations that survived to the end of the glaciation in the Central European mountains. Moreover, some diploid populations are still capable of parthenogenetic reproduction (Grebelyni 1996).

Analysis of mtCOI gene reveals that the parthenogenetic population of *C. staphylinus* is a separate subclade on the phylogenetic tree and includes two separate haplotypes with only one mutation between the distinct sequences (Figs. 2 and 3). In addition, the smallest values of haplotype and nucleotide diversity were observed for this population (Table 3). These facts support the suggestion that the parthenogenetic population of *C. staphylinus* has a clonal genetic structure. Usually, in sexual reproduction, mitochondrial genes are inherited exclusively from the mother (Travis 2000). The mtCOI gene shows rapid evolutionary rates, lack of introns and genetic recombination and allows to trace maternal lineage far back in time (Meyer 1993). In view of this, the clonal genetic structure from the observed parthenogenetic population points out that the males' genetic information was restricted during several generations.

The small genetic distance between the Vänern and Pääjärvi populations suggests that parthenogenesis in *C. staphylinus* in Pääjärvi might be of relatively recent origin. This idea was mentioned previously with statements of restricted distribution and high embryonic mortality in the parthenogenetic population (Sarvala 1979a). In some species of millipedes, parthenogenetic and dioecious populations were also mixed in one clade of the phylogenetic tree (Short and Vahtera 2017), suggesting that parthenogenesis may have evolved more recently as a specific adaptation to certain environmental conditions without changing the structure of nucleotide sequences.

As a rather big and fast-growing harpacticoid, *C. staphylinus* has relatively high food requirements, and thus prefers eutrophic environments (Sarvala 1979a,b; Hämäläinen and Karjalainen 1996). However, Lake Pääjärvi is oligotrophic, and food seems to be a limiting factor for the species. Under such conditions, the parthenogenetic mode of reproduction should give a selective advantage. Females of *C. staphylinus* from the parthenogenetic population of Lake Pääjärvi can copulate if males are present, but only few males are produced (Sarvala 1979a). Likewise, even other populations of *C. staphylinus* are often strongly female-biased (Lilljeborg 1902; Bevercombe 1973; Young 1974), or the numbers of males decrease during cold periods (Donner 1928; Smyly 1957).

Clearly, further studies of other populations of *C. staphylinus* (dioecious and possibly parthenogenetic), and possibly other species of this genus are needed to clarify both the taxonomy of this complex of species and the phenomenon of parthenogenesis in Canthocamptidae.

Conclusions

- 1) The harpacticoid species *C. staphylinus* shows a high level of genetic divergence between populations.
- 2) The parthenogenetic population of *C. staphylinus* from Lake Pääjärvi was genetically similar to the dioecious population from Lake Vänern, suggesting a relatively recent origin of parthenogenesis in this taxon.
- 3) The parthenogenetic population showed the smallest nucleotide and haplotype diversity, suggesting a clonal genetic structure in the population through the line of maternal inheritance of mitochondrial DNA.
- 4) The “northern” and “continental” clades of *C. staphylinus* can likely be considered as a complex of cryptic species.

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